AHAS Trp-574-Leu substitution in *Raphanus sativus* L.: screening, enzyme activity and fitness cost

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ABSTRACT

BACKGROUND: Feral radish (*Raphanus sativus* L.) is a problematic weed that has become resistant to AHAS (acetohydroxyacid synthase) inhibitor herbicides due to the Trp-574-Leu mutation. AHAS gene mutation that causes herbicide resistance may present negative pleiotropic effects on plant fitness. This study reports the effects of the Trp-574-Leu mutation on AHAS activity and reproductive traits of *R. sativus*.

RESULTS: Eight out of 17 feral radish accessions presented resistant individuals to metsulfuron-methyl from 0.5 to up to more than 90.0 % and all the resistant individuals analyzed showed the Trp-574-Leu mutation. Without herbicide selection, the AHAS activity of a susceptible accession

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was 3.2-fold higher than the resistant one. The resistant accession was > 9000-fold more resistant to metsulfuron-methyl and imazethapyr than the susceptible one. Under low intraspecific competition during two growing seasons, the AHAS resistant feral radish accessions showed 22 – 38 and 21 – 47 % lower seed number and yield per plant than the susceptible ones.

CONCLUSION: This is the first report of fitness cost associated with the AHAS Trp-574-Leu mutation in *R. sativus* populations. This fitness cost could reduce the frequency of the resistant allele without the herbicide selection.

Keywords

feral radish – resistance – Trp 574 Leu mutation – AHAS activity – pleiotropic effects

1. INTRODUCTION

Acetohydroxyacid synthase (AHAS), also known as acetolactate synthase (ALS), is the first enzyme in the biosynthesis of the branched chain amino acids valine, leucine and isoleucine [1]. AHAS is the common target site of five chemical families of herbicides: sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine (TP), pyrimidinyl-benzoates and sulfonyl-aminocarbonyl-triazolinone [1]. These herbicides inhibit the enzyme by binding within and obstructing the channel leading to the active site [2]. Since its introduction in the 1980s, AHAS inhibitor herbicides have been widely used due to their broad-spectrum weed control at very low rates, low environmental impact, low mammalian toxicity, wide crop selectivity and high control efficacy [1]. The intensive utilization of AHAS-inhibitor herbicides has resulted in a rapid evolution of AHAS resistant weed populations [3] and the increasing adoption of the no-till system together with a reduction in crop rotations have favored the increase of resistant weeds [4]. Nowadays, 159 weed species worldwide have become resistant to AHAS-inhibitor herbicides [5] which is mostly due to point mutations in the AHAS gene that reduce AHAS sensitivity to herbicides [3,6]. To date, 26 amino acid substitutions at eight positions of the AHAS gene (Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653 and Gly-654) have been identified that confer resistance to different AHAS inhibitor herbicides and in some of them, cross resistance to herbicides of two or more AHAS families [3,5,6]. The most commonly identified field-evolved amino acid substitutions that endow resistance to AHAS herbicides are at Pro-197 and Trp-574. In-field use of SU herbicides mostly select for Pro-197 mutations, while the use of SU and IMI herbicides mostly select for Trp-574 [3].

Evolutionary changes that adapt to a new environmental factor, such as herbicide resistance, may entail negative pleiotropic effects (also called cost of resistance) on fitness [7]. Herbicide target-site mutations can alter the enzyme functionality because some amino acid residues are important for herbicide binding but also for maintaining the AHAS activity [3,6,7]. Depending on the plant species

and the particular AHAS herbicide resistance-endowing amino acid substitution, studies had found a reduction [8–10], an increase [9,11–13] or no modification [13–16] in the AHAS activity. However, the alteration of the AHAS functionality does not necessarily translate into whole-plant pleiotropic effects [3]. For example, *Lolium rigidum*, *Raphanus raphanistrum* L. and *Kochia scoparia* with five, four and two AHAS resistance mutations, respectively, showed no significant impact on plant growth and competitiveness [3,9,11]. In these cases, resistance alleles are likely to remain in the population, even without herbicide selection, and in some cases may accumulate multiple mutations in the same plant [3]. Nevertheless, *Amaranthus powellii* with the Trp-574-Leu AHAS mutation showed a strong pleiotropic effect on plant morphology and anatomy, which resulted in significant reduction in growth and seed production [17]. An understanding of the fitness consequences of herbicide resistance alleles is important for predicting the evolutionary trajectory of herbicide resistance, and therefore, for conceiving strategies by which fitness costs can be manipulated to result in selection against resistance alleles [7].

Raphanus sativus L. (radish) is an ancient crop mainly domesticated for its edible roots. It is considered that spontaneous populations of radish (feral radish) found in America correspond to de-domesticated (feral) forms derived from the radish biotype [18]. Feral radish is a problematic weed in temperate zones of the Americas that can seriously reduce crop yields [18–20]. In Brazil, *R. sativus* interference reduced soybean plant height, branch length and yield [21]. Feral radish has an annual or biennial life cycle, it is self-incompatible, insect-pollinated [18] and it has developed resistance to herbicides, including AHAS inhibitors. The presence of AHAS-resistant *R. sativus* biotypes has been demonstrated in Brazil, Chile and Argentina [5,19,22,23]. In Argentina, *R. sativus* has been considered an invasive weed since the 1930s [24] and nowadays, it is a common weed in wheat, barley, oat, maize, canola, sunflower and several horticultural crops [20]. About 60 % of the southeast of Buenos Aires province is devoted to agriculture, which is mostly sown (up to 90 %)

under the no-till system and sulfonylureas herbicides are commonly used [25]. In this area, *R. sativus* is one of the weed species with the greatest increase since 1982 [25]. In 2011, some IMI-resistant sunflower fields were severely invaded with feral radish. The failure of IMI herbicides to control these feral radish populations was due to resistance to AHAS-inhibiting herbicides [23]. Populations showed resistance to five chemical families of AHAS inhibitor herbicides. The resistance was due to the Trp-574-Leu mutation in two of these biotypes. Since their initial report, the spread of *R. sativus* resistance to AHAS herbicides had rapidly increased, and in 2015, the Argentine No-Till Farmers Association (AAPRESID) reported the presence of AHAS resistant *R. sativus* in several districts in south central Buenos Aires province (AAPRESID 2015, http://www.aapresid.org.ar/rem/mapas-rem/).

It is unknown whether the Trp-574-Leu mutation, that causes resistance to several AHAS herbicides, presents some pleiotropic effects on AHAS activity and reproductive traits of *R. sativus* strains. Comparison of multiple field-collected resistant and susceptible populations provides some statistical power to account for the effect of differences in genetic background and helps determine the importance of different genetic backgrounds in influencing the fitness of resistance genes [26]. The aims of this study were to (1) detect and confirm whether diverse populations in the Pampas region are resistant to AHAS herbicides, and to (2) determine whether the AHAS gene Trp-574-Leu mutation can causes pleiotropic effects on AHAS activity and reproductive traits in *R. sativus* biotypes resistant to AHAS-inhibitors herbicides.

We detected eight *R. sativus* populations resistant to SU herbicide metsulfuron-methyl in the southeast of Buenos Aires, Argentina. In all the resistant individuals analyzed, a CAPS marker confirmed the AHAS-resistance-endowing amino acid substitution (Trp-574-Leu) like those found by Pandolfo et al. [23]. In addition, AHAS resistant feral radish accessions carrying the Trp-574-Leu mutation showed negative pleiotropic effects on AHAS activity and reproductive traits. This fitness

cost could reduce the frequency of the resistant allele during an absence of herbicide selection, at least where the population experiences low levels of intra/interspecific competition.

2. MATERIALS AND METHODS

2.1 Plant material

Mature pods of 17 feral *R. sativus* populations were collected in the Pampas, Argentina (Figure 1). AHAS resistant RSBA10 and RSBA3 and AHAS susceptible populations RSBA3f, RSBA1 and RSBA2 had already been characterized by Pandolfo et al. [23]. Both resistant populations were collected in agricultural fields under no-till production systems in southeastern Buenos Aires. Pods were crushed by hand or with a mortar to extract seeds minimizing physical damage. The seeds were cleaned and stored at room temperature until further use.

2.2 Screening test and resistance mutation genotyping

The response of *R. sativus* accessions RSLP2, RSBA7, RSBA11, RSBA12, RSBA13, RSBA14, RSBA15, RSBA16, RSBA17, RSBA18, RSBA19 and RSBA20 to metsulfuron-methyl was determined in 2015 and 2016. The RSBA10 and RSBA3 accessions were used as resistant controls. RSBA1, RSBA2 and RSBA3f accessions were considered as susceptible controls. At least 200 seedlings per accession were grown and metsulfuron-methyl applied at double the recommended rate (X = 6 g a.i. ha^{-1}) following the methods of Pandolfo et al. [23]. Surviving plants were recorded 35 days after herbicide application. Plants were classified as herbicide survivors if the growing point remained alive [27]. The experiments were arranged as a completely randomized design with four replications.

Genomic DNA of individual plants surviving the herbicide treatment was extracted and the presence of the Trp-574-Leu mutation was investigated by a CAPS marker following the procedure of Pandolfo et al. [23]. Three plants per accession for RSBA13, RSBA14 and RSBA17 and two for

RSBA11 were tested. The susceptible accession RSBA1 was included as a negative control and the resistant accession RSBA10 was used as a positive control. The PCR were performed with the gene-specific primers WR122F and W653R [16] to amplify the fragment of the AHAS gene encompassing position 574. The PCR amplified products were digested with *MfeI* restriction enzyme for detection of the Trp-574-Leu mutation. Digestion products were analyzed on 2 % agarose gels at 80 v for 1.5 h, stained with ethidium bromide, and documented by photographing under UV-C light (254 nm).

2.3 *In vitro* AHAS activity assay

In vitro AHAS activity of leaf tissue of susceptible (RSBA2) and resistant (RSBA10) feral radish accessions were determined following the procedure described by Yu *et al.*,[12] with negative controls for each dose according to Yu *et al.*,[11] with modifications. Leaf tissues (not including the petiole) of at least 10 young plants (2 – 3-leaf stage) in each extraction were ground to a fine powder in a mortar with liquid nitrogen and suspended in 5 mL g^{-1} fresh weight of buffer containing 100 mM HEPES [N(2-hydroxyethyl)-piperazine-N9-(2-ethanesulfonic acid)], pH 7.5, 200 mM sodium pyruvate, 20 mM MgCl2, 2 mM thiamine pyrophosphate (TPP), and 40 µM flavin adenine dinucleotide (FAD). Insoluble polyvinylpolypyrrolidone (PVPP) was added at the ratio of tissue:insoluble PVPP 6:1. The homogenate was filtered through four layers of gauze and centrifuged at 21,000 g for 15 min at 4 °C, and immediately used for enzyme activity assays. Crude extract (300 µl) and the same volume of distilled water or different herbicide concentrations (0.01 – 100,000 µM imazethapyr or 0.1 nM – 1,000 µM metsulfuron-methyl) were incubated at 37 °C for 60 min. SU herbicide metsulfuron-methyl (Nufarm, 60 % w/w i.a.) and IMI imazethapyr (Pivot, 10 % w/v i.a.) were used. Negative control was performed with denatured extract. The content of the reaction tube was divided into two aliquots of 250 µL. In one of the aliquots, the reaction was stopped by adding 100 µL of 5 N H_2SO_4 and incubated at 60 °C for 15 min to convert acetolactate

to acetoin. Acetoin-forming enzymes in plant tissues may interfere with the assay [28], thus the contribution of the direct formation of acetoin by non-AHAS enzyme activities was determined using 2.5 N NaOH in order to terminate the reaction, instead of H_2SO_4 in the second aliquot. Acetoin was quantified by a modified colorimetric assay [29] wherein the color was developed by adding 650 µL of 0.25% (w/v) creatine and 2.5% (w/v) α-naphthol prepared in 2.5 M NaOH just before use. The samples were vortexed, incubated at 60 °C for 15 min, allowed to cool and centrifuged at 25 °C for 5 min (11,000 g). Absorbance was measured spectrophotometrically at 530 nm. AHAS activity was calculated as the mean of three independent repetitions (independent extracts) and expressed as µM acetoin h^{-1} g^{-1} fresh weight.

The herbicide concentration causing 50 % inhibition of AHAS activity (I_{50}) was estimated with a non-linear log-logistic regression model. Goodness of fit of the data was evaluated using lack-of-fit *F*-tests at the 0.05 level of significance [30]. Dose-response data were fitted to the log-logistic model of three parameters [equation 1]:

Y = d/{1 + exp[b(log(x) - log(e))]} [equation 1];

where *e* denotes I_{50}, *d* the upper limit of the response and *b* denote the steepness of the dose-response curve around *e*. The lower limit value was fixed at 0 (three parameters), assuming that at high herbicide concentrations [31].

The extractable AHAS activity and multiple dose-response curves were compared between susceptible and resistant accessions using Student's t-test. Statistical analyses were performed using the *drc* package of R 3.3.1 statistical software [32]. The I_{50} values were used to calculate the resistance factor (RF), defined as the ratio between I_{50} of the resistant and susceptible accessions ($I_{50\,R}/I_{50\,S}$).

2.4 Fitness traits

Field experiments (S 38° 41′ 38″, W 62° 14′ 53″) were conducted in the Agronomy Department experimental field at the Universidad Nacional del Sur, Bahía Blanca, Argentina, to evaluate reproductive traits in resistant (R) and susceptible (S) feral radish accessions. R accessions were represented by two AHAS resistant accessions RSBA10 and RSBA3 with the Trp-574-Leu substitution characterized by Pandolfo et al. [23]. Four AHAS susceptible accessions RSBA2, RSBA3f, RSBA15 and RSBA16 obtained from a wide range of environmental conditions (Figure 1) were evaluated to reduce the effect of different genetic backgrounds [26]. Experiments were performed in two growing season between May 2016 – December 2016 (winter) and August 2016 – January 2017 (spring) to simulate feral radish growth dynamics, which is considered a facultative species 18 and two different cohorts are commonly observed in the agroecosystem.

Seedlings of each of the six accessions were established in plastic trays containing potting mix (Grow Mix Terrafertil), and grown in a greenhouse at 20 - 25 °C, watered twice daily and fertilized with a liquid fertilizer (Chase LI, grade 5-3-3). At the 3-4 leaf stage, 200 seedlings from each accession were transplanted to the experimental field in blocks, each consisting of six experimental units (one accession for each experimental unit). The experimental unit consisted of five rows spaced at 1 m, with 10 plants per row at 0.3 m intervals. Plants were drip irrigated and fertilized with 50 kg ha^{-1} diammonium phosphate at transplant and 120 kg ha^{-1} urea at the rosette state, for optimal plant growth. The experimental design was in randomized complete blocks with four replications. The assay was performed in the same manner in both growing seasons (winter and spring).

At the end of the growing season, plant height and branch number were measured in three successive plants in the middle of the central row of each experimental unit. The pods from these plants were then manually harvested and dried under laboratory conditions. Pods of each plant were counted and crushed by hand or using a mortar. Seeds were cleaned and weighed to obtain

the plant yield. The seed weight per plant was obtained by averaging the weight of four replicates of 100 seeds. The number of seeds per plant was estimated by dividing the seed weight per plant by the seed weight. The number of seeds per pod was estimated by dividing the seeds per plant by pods per plant. Data from the three plants per experimental unit were averaged for the statistical analysis.

To investigate the differences in plant height, branch number, pods per plant, seeds per pod, seeds per plant, seed weight and plant yield between susceptible and resistant biotypes we ran a linear mixed model based in restricted maximum likelihood estimation (REML) with PROC MIXED in SAS/STAT software (SAS University Edition, SAS Institute Inc., Cary, NC, USA). The growing season (winter and spring) and biotype (R and S) were considered as fixed effects, and block within growing season (winter and spring), accession (RSBA2, RSBA3, RSBA3f, RSBA10, RSBA15 and RSBA16) within biotype (R and S) and growing season*accession interaction were considered as random.

3. RESULTS

3.1 Screening test and resistance mutation genotyping

Eight populations showed surviving individuals at a double rate of metsulfuron-metyl (Table 1). All these populations were confined to three districts in southeastern Buenos Aires Province (Figure 1). The susceptible RSBA3f population was found in the field margin on the farm where the resistant RSBA3 population was found. The susceptible RSBA7 accession was collected less than 50 km from resistant populations, whereas the remaining susceptible populations were found more than 200 km from resistant populations (Figure 1). The population size was highly variable for both the susceptible and resistant populations (Table 1).

Digestion of the PCR products with *MfeI* restriction enzyme revealed that the susceptible individuals had the wild-type (WT) allele represented by the undigested fragment of 0.5 kb. All the resistant individuals evaluated showed the mutant allele represented by the digested fragments of 0.29 and 0.23 kb, indicating the presence of the mutation Trp-574-Leu.

3.2 AHAS activity

In the absence of AHAS herbicides, the extractable AHAS activity of susceptible feral radish RSBA2 (731.6 µM acetoin h^{-1} g^{-1}) was 3.2-fold higher (t = 5.79, P = 0.0044) than the resistant RSBA10 (226.4 µM acetoin h^{-1} g^{-1}).

Herbicide sensitivity to the AHAS activity from R-RSBA10 and S-RSBA2 plants was determined using IMI imazethapyr and SU metsulfuron-methyl herbicides. The I_{50} values of the AHAS-susceptible accession were 1.01 ± 0.29 µM for imazethapyr and 2.76 ± 0.77 nM for metsulfuron-methyl (Figure 2). However, the I_{50} values of the resistant accession were 9,199 ± 1,676 µM for imazethapyr and 26,655 ± 8,684 nM for metsulfuron-methyl (Figure 2). Based on the I_{50}R ⍰S ratio the Trp-574-Leu mutation was 9,109 and 9,663-fold resistant to imazethapyr and metsulfuron-methyl, respectively.

3.3 Fitness traits

The second growing season showed 48 % lower plant height, 53 % fewer pods per plant, 55 % lower seeds per plant, 19 % lower seed weight and 62 % fewer plant yield than the first growing season, but there was no significant difference in seeds per pod between the growing seasons (Table 2). There was no significant growing season*accession interaction in plant height, pods per plant, seeds per pod, seeds per plant, seed weight and plant yield, therefore data from the two growing seasons were pooled (Table 2). However, there was significant growing season*accession interaction in branch number, therefore the growing seasons were separately evaluated (Table 2).

No significant differences were found in plant height, branch number, seeds per pod and seed weight between S and R biotypes (Table 2). However, R accessions had 17 – 24 % fewer pods per plant, 22 – 39 % fewer seeds per plant, and 21 – 47 % lower plant yield than the S accessions (Table 2, Figure 3). In addition, the resistant accession RSBA3 had 24 % fewer pods per plant, 31 % fewer seeds per plant and 39 % lower plant yield than their susceptible counterpart RSBA3f (Table 1; Figure 3).

4. DISCUSSION AND CONCLUSIONS

We found eight out of 17 *R. sativus* accessions with different levels of resistance to SU herbicide metsulfuron-methyl in the southeast of Buenos Aires province, Argentina (Figure 1, Table 1). In all of the resistant individuals tested, a CAPS marker confirmed the AHAS-resistance-endowing amino acid substitution (Trp-574-Leu) as those found by Pandolfo et al. [23] (RSBA3 and RSBA10). This mutation produced an enzyme >9000-fold more resistant than the enzyme without the substitution. However, the Trp-574-Leu mutation showed negative pleiotropic effects on AHAS activity in the absence of herbicide selection, which was probably the cause of the reproductive fitness cost found, under low levels of competition.

In southeastern Buenos Aires, more than 90 % of the agricultural area is sown in no-till production systems mainly with wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), soybean [*Glycine max* (L.) Merr.] and sunflower (*Helianthus annuus* L.) [25]. Imidazolinone-resistant sunflower cultivars (Clearfield® and Clearfield Plus®) represent more than 60 % of the sown area of sunflower (ASAGIR 2017, http://www.asagir.org.ar/) and sulfonylurea is the most frequently used herbicide family. Metsulfuron-methyl is the most commonly applied herbicide for controlling broadleaf weeds in wheat and barley [25]. In turn, crop rotations with forage pastures have been reduced in this area, leading to an increase in continuously cultivated fields and monocultures, with the

consequent use of the same herbicides. This intensive selection pressure, caused by the massive use of SU and IMI herbicides, has probably resulted in selection for *Amaranthus palmeri*, *Hirschfeldia incana*, *Lolium multiflorum* and *R. sativus* plants resistant to AHAS herbicides (AAPRESID 2015, http://www.aapresid.org.ar/rem/mapas-rem/), the latter with the Trp-574-Leu amino acid substitution [3,23]. The AHAS herbicide resistance mechanism in *Amaranthus palmeri, Hirschfeldia incana* and *L. multiflorum* biotypes from Argentina it is still unknown.

The six new feral radish accessions resistant to AHAS inhibitors, with the Trp-574-Leu mutation, were found close to those reported by Pandolfo et al. [23]. The RSBA11, RSBA13, RSBA14, RSBA17 and RSBA20 populations were found less than 40 km from the RSBA10 population, and the RSBA19 population was found in a field close (< 5 km) to the RSBA3 population (Figure 1). *R. sativus* is a self-incompatible and insect-pollinated species [18] and AHAS resistance alleles are dominant [3] hence gene flow between nearby populations could facilitate the propagation of feral radish resistant to AHAS [33]. In addition, in this area sowing and harvesting are mostly carried out with hired machinery, thus the use of the same agricultural equipment in different fields within the area could have facilitated the dispersion of resistant *R. sativus* seeds around the fields [34]. Moreover, the selection of the Trp-574-Leu mutation in different populations of *R. sativus* at the same time as a result of independent events cannot be ruled out. On the other hand, the low proportion of AHAS resistant plants in the RSBA11 feral radish populations could be attributed to absence of selection by herbicides, a recent mutational event or recent gene flow from resistant plants nearby.

The inhibition of *in vitro* AHAS activity of susceptible feral radish in response to metsulfuron-methyl and imazethapyr was >9000-fold higher than the resistance with the Trp-574-Leu mutation, which probably indicates that the resistance is due to reduced AHAS sensitivity to both herbicides. This value cannot be taken as the herbicide concentration inhibiting AHAS activity *in*

vivo because nothing is known about herbicide uptake, translocation, and metabolism. However, these results are consistent with those observed by Pandolfo et al. [23] for the same accession and herbicides at the whole plant level, although the resistance factor values are not comparable [8]. McCourt et al. [2] have found that the Trp-574-Leu mutation in the AHAS gene changes the shape of the herbicide binding site and it results in the loss of several interactions that produce an enzyme highly resistant to AHAS herbicides. This mutation has been found to provide resistance to AHAS herbicides in at least 36 species [5]. Weed species with the Trp-574-Leu mutation have shown variable levels of resistance *in vitro*. Massa et al. [35], Yu et al. [11] and Cross et al. [15] reported a level of resistance > 8333, > 1333 and 10 for *Apera spica-venti, Lolium rigidum,* and *Poa annua*, respectively, in response to different SU herbicides. In addition, Chen et al. [13] showed a factor of resistance of 268 for *Amaranthus retroflexus* in response to IMI imazethapyr.

Of the 36 species that are resistant to AHAS herbicides due to the substitution of the Trp-574-Leu amino acids [5], only a few species have been tested for associated pleiotropic effects [7]. AHAS herbicides do not bind to the active site but near to it, inhibiting AHAS activity by blocking substrate access. Although, target-site mutations conferring herbicide resistance may not reduce AHAS activity, the Trp-574-Leu mutation could be involved not only in the herbicide binding, but also in maintaining AHAS activity, so its conservation would be necessary and the cost of resistance is otherwise expected [3]. We found significantly lower extractable *in vitro* AHAS activity in the resistant population than in the susceptible one. This lower AHAS activity associated with the Trp-574-Leu mutation is not in agreement with the results of Yu et al. [11] and Chen et al. [13] who found higher activity in the enzyme in *Lolium rigidum* and *Amaranthus retroflexus*. In addition, Cross et al. [15] found no changes in AHAS activity of *Poa annua*. However, Li et al. [9] reported similar results in an AHAS resistant population of the wild relative *R. raphanistrum* with Trp-574-Leu mutation. However, the difference in AHAS activity observed in the present study between

resistant vs. susceptible accessions was greater as that found in *R. raphanistrum* [9]. As discussed above, the Trp-574-Leu mutation may cause increased, reduced or unchanged AHAS activity in different plant species. The AHAS enzyme is composed of catalytic and regulatory subunits, and the latter, without AHAS activity itself, stimulates activity in the catalytic subunit and confers sensitivity to feedback inhibition by branched chain amino acids [1,3]. The regulatory mechanisms that govern enzyme AHAS activity and how these may act differently in the same mutation in different plant species remain relatively unknown. In addition, difficulty in isolating and maintaining AHAS activity in susceptible vs. resistant plants has been observed for many amino acid substitutions that confer resistance [11]. This could explain the lack of differences in the extractable AHAS activity found between resistant and susceptible accessions in several assays.

The lower production of the branched-chain amino acid due to decreased enzyme AHAS activity in the resistant vs. susceptible could impact on protein synthesis and this could affect metabolism at some key points in the plant lifecycle leading to a lower plant fitness. It has not always been possible to correlate the alteration of AHAS activity with whole-plant pleiotropic effects [7]. However, our results show an association between AHAS activity and fitness cost in *R. sativus* accessions. In this study, we found no significant differences in plant height, branch number, seeds per pod and seed weight between resistant and susceptible biotypes of *R. sativus*. However, the susceptible biotypes showed higher pods per plant than the resistant ones, which resulted in lower seed number and yield per plant in the resistant biotypes compared to the susceptible ones. Similarly, the Trp-574-Leu mutation showed alteration in the leaf morphology and reduction vegetative and reproductive growth in populations of *Amaranthus powellii* [17]. This alteration in the leaf morphology could occur because the Trp-574-Leu mutation interferes with the normal C4 expression in developing leaves [17]. Nevertheless, *R. sativus* is a C3 species and so far, there is no evidence that the Trp-574-Leu mutation affects the expression of leaf development. In addition,

the relative growth rates at different harvest intervals between susceptible and resistant biotypes of *Poa annua* with the Trp-574-Leu mutation were similar. However, the resistant biotype produced more inflorescences and seeds per plant in comparison with the susceptible one [15]. On the other hand, the AHAS resistant Trp-574-Leu mutation did not impose negative pleiotropic effects on vegetative growth in isolated and competing plants, photosynthesis and resource-competitive ability in the wild relative *R. raphanistrum* [9]. Additionally, it is important to highlight that the plants in the first growing season had greater height, branch number, pods per plant, seed weight, seeds and yield per plant than in the second growing season (Table 2). Therefore, these differences could be translated into greater competitive ability and greater seed input into the seedbank of feral radish plants in the first growing season (winter) vs. plants of the second growing season (spring).

The comparison of multiple field-collected resistant and susceptible populations was the strategy to address the variable effect of genetic background on resistant cost but because only two AHAS Trp-574-Leu resistant accessions were used [23], these results should not be generalized. On the other hand, there was not significant effect accession within biotype (resistant and susceptible) (Table 2) for any trait, this indicate reduced variability between accessions within biotype. In addition, the resistant accession RSBA3 was significantly less fit that their counterpart RSBA3f (Figure 3). The latter accession was collected in the same location that the resistant one but over the uncontrolled edges of the field. Therefore, it can be assume that both accessions had similar genetic background. Nevertheless, the possibility that the pleiotropic effects that was observed is not caused by the Trp-574-Leu mutation but only correlated with it cannot be completely ruled out. Further experiments are required to fully understand the interaction between this mutation and fitness traits i.e. compare susceptible and resistant plants with the same genetic background.

The fate of an adaptive mutation, e.g., the AHAS Trp-574-Leu resistance mutation, might primarily depend on its initial frequency in the population [36]. However, its evolutionary trajectory depends on a balance between the weed breeding system, dominance of the resistant allele, resistance cost and herbicide management practices [37,38]. *R. sativus* is a cross-pollinated species [18] and the AHAS resistant allele is dominant [3], two characteristics that would increase the frequency of the resistant allele in a population. However, our study under low intra/interspecific competition suggests that the AHAS gene resistance endowing mutation Trp-574-Leu imposes negative pleiotropic effects on reproductive traits of feral *R. sativus*, which would act as a counterbalance in resistance evolution [37]. Continuous selection by herbicides in cross-pollinated species favors the formation of resistant homozygotes [37]. Accordingly, we have found populations with high levels of resistance to metsulfuron-methy where there is high selection pressure in field edges or centers (RSBA3, RSBA10, RSBA13, RSBA14, RSBA17, RSBA19 and RSBA20). Nevertheless, the resistant cost found in this study could reduce the frequency of the resistant allele in the absence of herbicide selection. In accordance with this, we have found populations with null/low levels of resistance in fencerows or field margins (RSLP2, RSBA1, RSBA2, RSBA3f, RSBA7, RSBA11, RSBA12, and RSBA18), where the herbicide selection is absent.

Gene flow between these two population classes could change the dynamics of resistant alleles [37]. Gene flow from populations with null/low resistance level could act as a refuge, delaying the resistance evolution in populations with high levels of herbicide selection. However, when gene flow occurs in the opposite direction it results in increasing the resistance frequency in habitats without herbicide selection [37]. This gene flow will be greater when the populations are closer and from larger populations to smallers one [38].

Our study suggests that under low intra/interspecific competition, the AHAS gene resistance endowing mutation Trp-574-Leu can impose negative pleiotropic effects on reproductive traits in

feral *R. sativus*. This fitness cost could reduce the frequency of the resistant allele in the absence of herbicide selection. To reinforce these results, future research should focus on determining the fitness cost of AHAS-resistant Trp-574-Leu under competition with crops and/or using susceptible and resistant plants with the same genetic background.

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Table 1. Characteristics of the populations of *Raphanus sativus* used in the present study.

Reference code	Collection date	Crop and field position	Population size	Resistance level*
RSLP2	Jan-13	Fencerow	>100	0.0
RSBA1[2]	Nov-08	Canola field margin	>10000	0.0
RSBA2[2]	Nov-08	Fencerow	>100000	0.0
RSBA3	Nov-08	Canola field edge	10-50	81.1 ± 5.1
RSBA3f[1]	Nov-08	Canola field margin	>1000	0.0
RSBA7	Dec-09	Fencerow	1-10	0.0
RSBA10	Apr-11	Sunflower field centre	>10000	94.3 ± 2.0
RSBA11	Dec-11	Fencerow	>100	0.5 ± 0.3
RSBA12	Feb-12	Maize field margin	>1000	0.0
RSBA13	Mar-14	Field edge	50-100	89.5 ± 3.6
RSBA14	Dec-14	Barley field edge	1-10	65.2 ± 4.6
RSBA15[3]	Mar-15	INTA Experimental station	>10000	0.0
RSBA16	May-15	Sunflower field centre	n.d.	0.0
RSBA17	Nov-15	Barley field centre	>10000	90.5 ± 6.2
RSBA18	Apr-16	Soybean field margin	500-1000	0.0
RSBA19	Apr-16	Barley field edge	>1000	94.9 ± 1.1
RSBA20	Apr-16	Soybean field edge	10-50	92.9 ± 0,8

*Percentage of plants resistant to metsulfuron-methyl (Screening test).

[1] RSBA3f population was collected in the field margin on the farm where the RSBA3 population was found.

[2] They were collected in areas without any herbicide application.

[3] It was collected in a field of National Institute of Agricultural Technology (INTA), Hilario Ascasubi Agricultural Experiment Station, Hilario Ascasubi, Buenos Aires, Argentina.

Table 2. Restricted maximum likelihood (REML) table for fitness traits of *Raphanus sativus* resistant and susceptible to AHAS herbicides.

Effect			Plant height		Branch number (winter)		Branch number (spring)		Pod number plant		Seed number pod		Seed number plant		Seed weight		Yield plant	
Fixed	$^1DF_{NUM}$	$^1DF_{DEN}$	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
Growing season	1	5	295.21	<.0001	-	-	-	-	42.51	0.0013	0.96	0.3732	48.16	0.0010	19.10	0.0072	78.34	0.0003
Biotype	1	4	0.18	0.6945	0.41	0.5558	0.05	0.8359	8.01	0.0473	3.22	0.1472	15.73	0.0166	2.35	0.2002	16.97	0.0146
Random			*Z*-value	*P*	*Z*-value	P	*Z*-value	P	*Z*-value	*P*	*Z*-value	*P*	*Z*-value	*P*	*Z*-value	*P*	*Z*-value	*P*
Block (growing season)			0.84	0.2013	0.25	0.4006	0.89	0.1870	0.95	0.1702	0	.	0.71	0.2383	0.77	0.2217	0.65	0.2579
Accession (biotype)			0	.	0.98	0.1631	1.23	0.1089	0	.	0.80	0.2130	0	.	0.99	0.1612	0.34	0.3677
Growing season*accession			1.19	0.1174	-	-	-	-	0	.	0	.	0.41	0.3415	0.51	0.3061	0.26	0.3978
Residuals			3.87	<.0001	2.74	0.0031	2.74	0.0031	4.42	<.0001	4.53	<.0001	3.87	<.0001	3.87	<.0001	3.87	<.0001

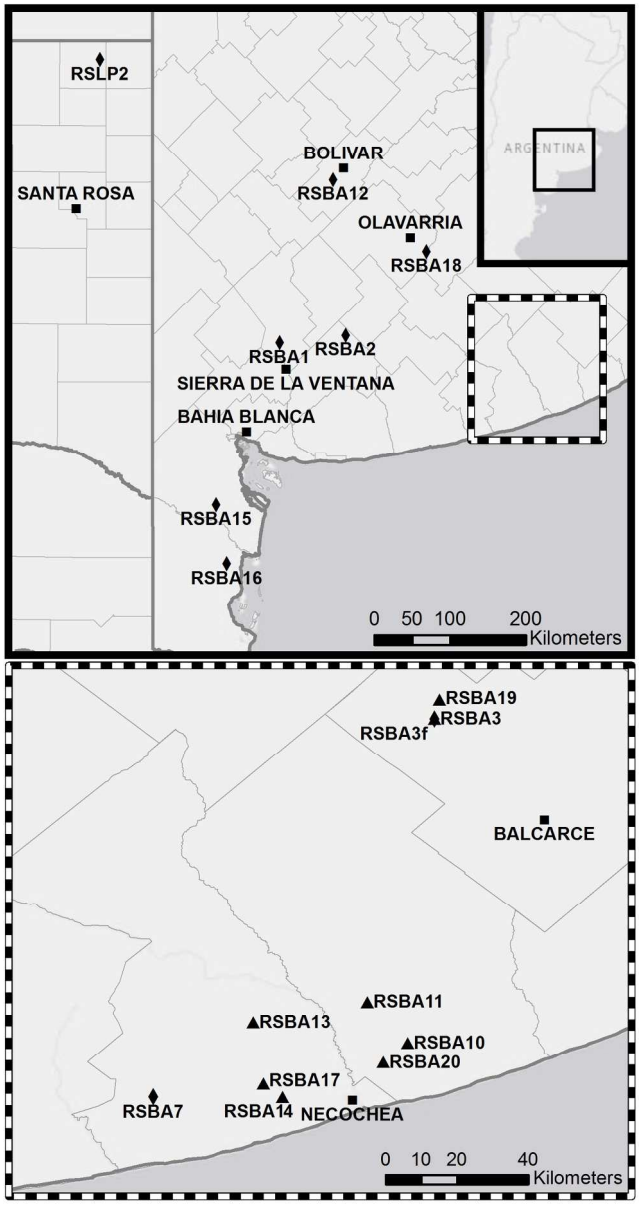
Linear mixed model was performed for seven fitness traits for six accessions of *Raphanus sativus* within two biotypes (susceptible and resistant), with four and two accessions within susceptible and resistant biotypes, respectively. Data are based in two growing seasons, except the variable 'branch number' that it showed growing season*accession interaction, therefore we showed its results for each growing season (winter and spring).

$^1DF_{NUM}$ and DF_{DEN} correspond to all variables studied.

Figure 1. Sites where *Raphanus sativus* accessions were found. Symbols indicate resistant (▲) and susceptible (♦) accessions and cities (■) used as reference. Solid inset shows populations in the Pampas, Argentina. Dash inset shows populations in the southeast of Buenos Aires province.

Figure 2. In vitro inhibition of AHAS activity by AHAS-inhibiting herbicides (a, imazethapyr; b, metsulfuron-methyl) in the resistant (RSBA10, Trp-574-Leu) and susceptible (RSBA2, wild type) *Raphanus sativus* accessions. AHAS activity was expressed as a percentage of activity in the absence of herbicide. Hundred percent AHAS activity was 584.0 ± 64.4 and 180.4 ± 26.5 µM acetoin h-1 g-1 for RSBA2 and RSBA10, respectively (a), and 879.2 ± 327.0 and 272.4 ± 36.3 µM acetoin h-1 g-1 for RSBA2 and RSBA10, respectively (b). Symbols are the mean of three replicates.

Figure 3. Pods per plant (a), seeds per plant (b) and plant yield (c) from six accessions of *Raphanus sativus* divided in two biotypes, susceptible (RSBA2, RSBA3f, RSBA15 and RSBA16) and resistant (RSBA3 and RSBA10) for AHAS resistance mutation Trp-574-Leu. Data are the average of two growing seasons. Values are mean (n = 24) and vertical bars represent ± 1 standard errors.



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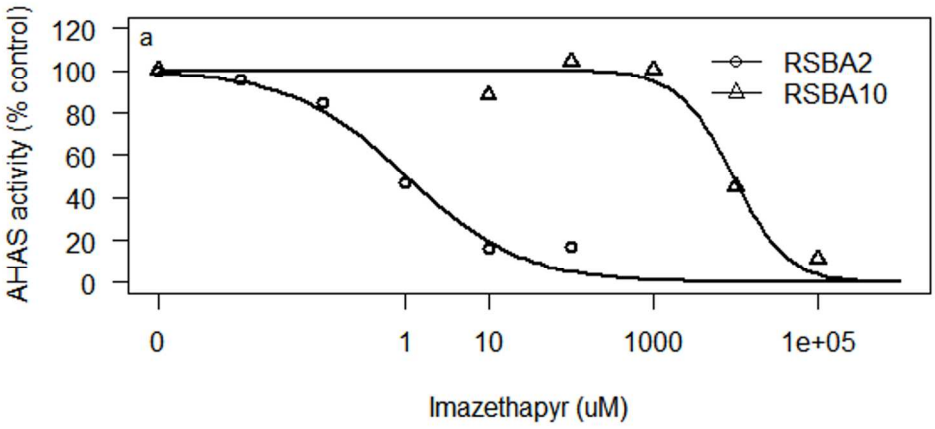

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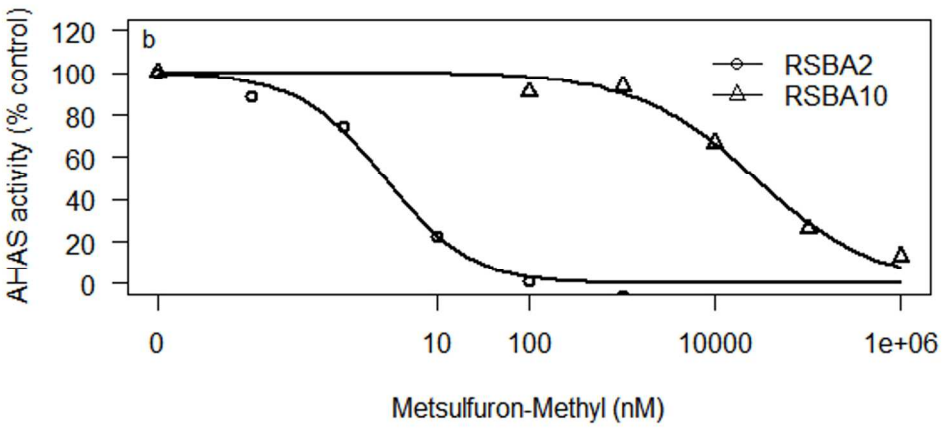

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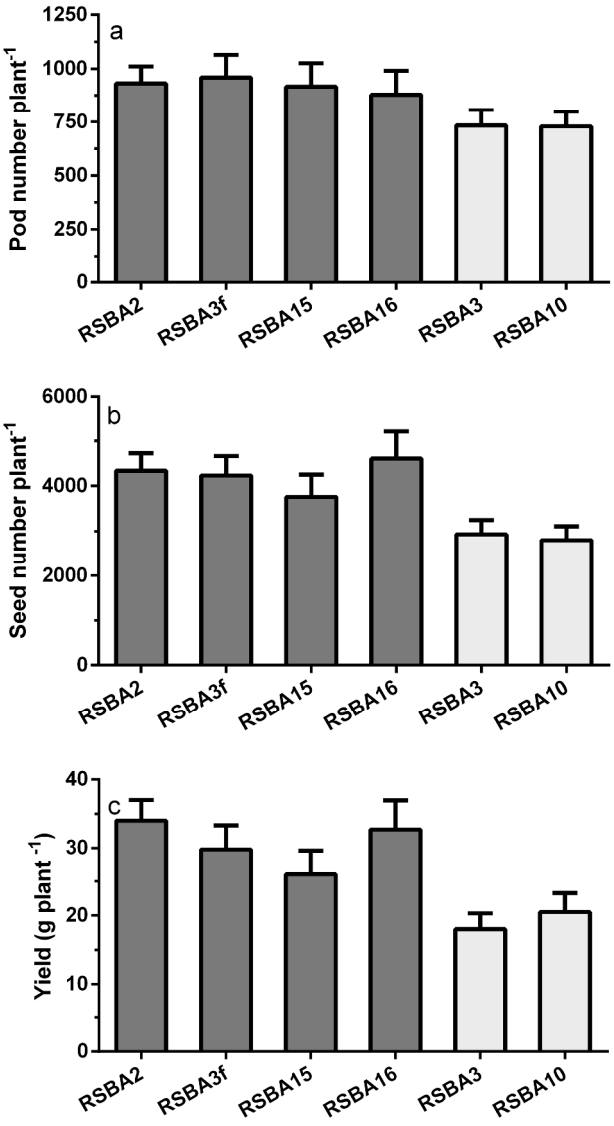

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